Subunit interactions influence the biochemical and biological properties of Hsp104

Subunit interactions influence the biochemical and biological properties of Hsp104

Eric C. Schirmer*†, Danielle M. Ware‡§, Christine Queitsch*§, Anthony S. Kowal‡, and Susan L. Lindquist*‡¶

*Department of Molecular Genetics and Cell Biology and ‡Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637

Contributed by Susan L. Lindquist, November 30, 2000

Point mutations in either of the two nucleotide-binding domains (NBD) of Hsp104 (NBD1 and NBD2) eliminate its thermotolerance function *in vivo*. *In vitro*, NBD1 mutations virtually eliminate ATP hydrolysis with little effect on hexamerization; analogous NBD2 mutations reduce ATPase activity and severely impair hexamerization. We report that high protein concentrations overcome the assembly defects of NBD2 mutants and increase ATP hydrolysis severalfold, changing V_{max} with little effect on K_m. In a complementary fashion, the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate inhibits hexamerization of wild-type (WT) Hsp104, lowering V_{max} with little effect on K_m. ATP hydrolysis exhibits a Hill coefficient between 1.5 and 2, indicating that it is influenced by cooperative subunit interactions. To further analyze the effects of subunit interactions on Hsp104, we assessed the effects of mutant Hsp104 proteins on WT Hsp104 activities. An NBD1 mutant that hexamerizes but does not hydrolyze ATP reduces the ATPase activity of WT Hsp104 *in vitro*. *In vivo*, this mutant is not toxic but specifically inhibits the thermotolerance function of WT Hsp104. Thus, interactions between subunits influence the ATPase activity of Hsp104, play a vital role in its biological functions, and provide a mechanism for conditionally inactivating Hsp104 function *in vivo*.

The HSP100/Clp family of chaperone proteins plays a wide variety of important cellular roles in different organisms, including survival of environmental stress, regulation of genetic competence, transposition, proteolysis, and control of a protein-based genetic element (prion). These seemingly unrelated roles are unified by a common remarkable biochemical mechanism; the proteins promote the disassembly of aggregated proteins and higher-order protein complexes (reviewed in refs. 1 and 2). Several biochemical properties are shared by HSP100 proteins and are required for their biological functions, suggesting that these properties are important to the complex phenomenon of protein disassembly. These properties include an ATP-hydrolyzing activity (3–10) and the ability to self-assemble into oligomers, primarily hexamers (11–13).

A member of the HSP100 family from *Saccharomyces cerevisiae*, Hsp104, is critical for survival after exposure to extreme temperatures (50°C) (14) or high concentrations of ethanol (20%) (15). These stresses cause protein denaturation, and Hsp104 promotes survival by facilitating the resolubilization of heat-damaged, aggregated proteins (reviewed in refs. 1 and 2). At normal temperatures, Hsp104 plays a critical role in the inheritance of the novel proteinaceous genetic element [*PSI*+] (often called a yeast prion), an ordered aggregate of the translation terminator, Sup35 (16).

Hsp104 is a member of the class 1 HSP100 proteins, with two distinct but highly conserved nucleotide-binding domains (NBDs) (1, 17). NBD1 and NBD2 of Hsp104 show only 22% amino acid identity with each other, yet each shares 40–60% identity with the corresponding domains of their *Escherichia coli* relatives, ClpA and ClpB (18). Although NBD1 and NBD2 are very different from each other, both contain classic Walker-type consensus sequences for the P-loop (19) that resemble those of NSF and the P-type transporter protein families. ATP promotes the assembly of Hsp104 into hexamers (11), and Hsp104 hydrolyzes ATP with a high degree of specificity (10). The mechanism by which Hsp104 performs its functions is not understood. However, both its ATPase activity and its oligomerization properties seem to be essential. Point mutations that perturb these functions (10, 11) eliminate the ability of Hsp104 to provide thermotolerance (20) and interfere with the normal metabolism of the [*PSI*+] element (16).

Previously, we reported that canonical P-loop residue mutations in NBD1 of Hsp104 virtually eliminate ATP hydrolysis but have little effect on oligomerization. In contrast, analogous mutations in NBD2 inhibit but do not eliminate ATP hydrolysis and severely impair oligomerization (10, 11). Based upon the crystal structures and mutational analysis of other Walker-type ATP binding sites (21), these mutations were chosen to inhibit ATP binding without perturbing the general structure of the domain. The related *E. coli* protein, ClpA, is similar to Hsp104 in that analogous point mutations in NBD1 and NBD2 differently affect oligomerization and ATP hydrolysis (22, 23). Curiously, however, the effects of the mutations are reversed from those reported for Hsp104 (10). Either the two domains have independent functions (one responsible for ATP hydrolysis, the other for oligomerization) and these functions have switched in the two proteins during the course of evolution or both domains contribute to ATP hydrolysis and oligomerization in a complex, interdependent manner, and other differences between the two proteins idiosyncratically cause analogous mutations to perturb one of these characteristics more than the other. Indeed, mutations that abolish oligomerization in Hsp104 or in ClpA also reduce ATP hydrolysis (10, 22, 23), suggesting a relationship between oligomerization and ATP hydrolysis. However, no kinetic information is available relating these properties in any HSP100 protein nor relating subunit interactions to biological function.

Here, we demonstrate that interactions between Hsp104 subunits increase the ATPase activity of Hsp104 in a cooperative fashion. We also report that Hsp104 proteins that carry point mutations in NBD1 or NBD2 profoundly alter the activities of wild-type (WT) Hsp104 in mixed oligomers and do so in different ways both *in vitro* and *in vivo*. The data support previous suggestions of functional distinctions in the two NBDs, illustrate the importance of proper subunit interactions in establishing the functional state of Hsp104, and provide different ways to alter that state.

Materials and Methods

Plasmids. Point mutations in *HSP104* were produced and placed into expression vectors as described (10). Mutant *HSP104* coding

Abbreviations: NBD, nucleotide-binding domain; WT, wild-type; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

†Present address: Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, 92037.

§D.M.W. and C.Q. contributed equally to this work.

¶To whom reprint requests should be addressed. E-mail: s-lindquist@uchicago.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.031568098. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.031568098

sequences were inserted behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter in pRS305 for integration at the *LEU2* locus. The WT *HSP104* coding sequence was placed under Gal1 regulation in pRS316 (cen6/ars4, *URA3*; 104_bU). pHCA/GAL4(1–93).ER.VP16, which encodes a β-estradiol-activated inducer of Gal1-regulated genes (24), was the gift of D. Picard. Late in the course of these studies, we found that one of the mutants, K218T, had acquired an additional mutation producing K218T:A315T. This mutant behaved similarly to K218T alone when retested for assembly and ATP hydrolysis.

Strains. The integration constructs described above and their parent vector, pRS305 (cleaved with *Afl*II), were integrated into yeast strain MD104–1U in which 80% of the *HSP104* coding sequence is deleted. These strains were subsequently transformed with two plasmids: pHCA/GAL4(1–93).ER.VP16 and either pRS316 or 104_bU. This generated strains with the following combinations of integrated glyceraldehyde-3-phosphate dehydrogenase-regulated (constitutive)/plasmid β-estradiol-regulated genes: vector/vector, K218T:A315T/vector, vector/WT, K218T:A315T/WT, K620T/vector, and K620T/WT.

Protein Purification. Hsp104 was purified from yeast and *E. coli* as described (11, 25). To compare mutant and WT, proteins were first dialyzed against and concentrated in 20 mM Hepes, pH 7.5/140 mM KCl/15 mM NaCl/10 mM $MgCl_2$/2 mM DTT using Ultrafree-15 centrifugal filter devices with M_r cut-off at 30,000 Da (Millipore). After concentrations were determined (25), proteins were diluted in the same buffer.

ATPase Assays. To relate ATP hydrolysis to oligomerization, both types of assay were performed in reaction buffer 1 (20 mM Hepes, pH 7.5/140 mM KCl/15 mM NaCl/10 mM $MgCl_2$). All reactions included 5 mM ATP (pH 7.5) except for K_m experiments, where the reaction buffer, containing 5 mM $MgCl_2$, included Mg-ATP (0.01 to 40 mM). ATP and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were adjusted to pH 7.5.

All assays shown were performed, as described (10), for times at which the phosphate released was linear for the assay system. Released P_i was quantified with malachite green (26), and A_{650} was determined with a Molecular Devices V_{max} kinetic microplate reader with SOFTMAX software. Values were calibrated against KH_2PO_4 standards and corrected for phosphate released in the absence of Hsp104. For each point, at least three and typically six independent assays were used to calculate the mean and standard deviation. The curves shown and K_m and V_{max} values (500 μM to 25 mM ATP) were generated by using least squares fitting to the Michaelis–Menten equation with the KALEIDAGRAPH graphics program (Synergy Software, Reading, PA). Similar values were obtained when Lineweaver–Burke and Eadie–Hofstee plots were used. For calculation of the Hill coefficient (10 μM to 40 mM ATP), nonlinear curves fitted to the Michaelis–Menten equation were generated by plotting the rate of hydrolysis μg^{-1} protein versus the concentration of ATP using the nonlinear allosteric kinetic equation of the GRAFIT 4 graphics program (Erithacus Software, Surrey, U.K.).

Similar results were obtained with a second assay for which 5 μCi of [α-^{32}P]ATP (3,000 MBq) was added per 25-μl reaction; reactions were stopped by addition of formic acid to 1.5 M; ATP and ADP were resolved on Baker-flex cellulose PEI plates using 0.25 M LiCl, 0.375 M Tris, 1 M formic acid; and the percentage of ATP hydrolyzed was determined from comparison of the intensity of ADP and ATP spots with a Molecular Dynamics PhosphorImager using IMAGEQUANT software.

Cross-Linking Assays. Wild-type and mutant proteins dialyzed against reaction buffer 1 with 2 mM DTT were adjusted to the indicated concentrations in reaction buffer 1 containing DTT (2 mM) with or without ATP (5 mM). Assay volumes were 200 μl for 0.01 mg/ml, 120 μl for 0.02 mg/ml, 100 μl for 0.05 mg/ml, 40 μl for 0.1 mg/ml, and 10 μl for 0.5 mg/ml reactions. Reactions were incubated at 37°C for 10 min, glutaraldehyde was added (final concentration 0.01%), and cross-linking was terminated as described (11). Conditions were the same for experiments with CHAPS except that 3 mM ATP was used instead of 5 mM.

Thermotolerance Assays. Cells were grown to equal logarithmic-phase densities, the 104bU plasmid was induced with 100 nM β-estradiol for 2 h, and aliquots were moved directly to 50°C for 4 min and then plated on noninducing media to determine survival rates as described (27). During the heat shock, additional aliquots were taken for protein quantitation by Western analysis as described (27), except that immune complexes reacted with ^{125}I-labeled protein A were quantified by using a Molecular Dynamics PhosphorImager and IMAGEQUANT software.

Results

The Relationship Between Hexamerization and Protein Concentration. In buffers that approximate physiological salt concentrations, the assembly of Hsp104 into hexamers is driven by the addition of adenine nucleotides (11). In the absence of added nucleotide, Hsp104 forms hexamers if salt concentrations are low (i.e., 20 mM; ref. 10). We sought additional conditions that would promote assembly in the absence of ATP and found that increasing the protein concentration in physiological ionic strength buffers did so (Fig. 1*A*). When Hsp104 was cross-linked with glutaraldehyde at a protein concentration of 0.1 to 0.5 mg/ml, most of the protein migrated on SDS/PAGE as a hexamer (this is apparent from counting the steps in the ladder of species obtained by cross-linking with glutaraldehyde at shorter incubation times). When Hsp104 was cross-linked at a concentration of 0.01 mg/ml, most of the protein migrated as a monomer. When ATP was present, cross-linking yielded hexamers at all of these protein concentrations. Gel filtration chromatography and multiangle light scattering confirmed that Hsp104 was hexameric at high protein concentrations and existed as a combination of monomers and dimers at low protein concentrations (D. Hattendorf and S.L, unpublished results). For simplicity, we refer to the latter state as "unassembled."

We next asked if high protein concentrations could overcome the oligomerization defects of Hsp104 NBD2 point mutants (Fig. 1*B*), which fail to assemble at ATP concentrations at which the WT protein is fully assembled (10, 11). One of these, K620T, carries a threonine substitution in the conserved lysine of the P-loop motif that interacts directly with the β- and γ-phosphates of bound nucleotide in other nucleotide-binding proteins with this motif (21); the other, G619V, is a glycine to valine substitution in residue 619 (Fig. 1*B*). Previously, only K620T was tested at a protein concentration of 0.01 mg/ml. It was unassembled even in the presence of 5 mM ATP (10, 11). Here, for both of these mutants, some assembly was observed at a protein concentration of 0.05 mg/ml; at 0.5 mg/ml, nearly all of the protein was hexameric (Fig. 1*C* and data not shown). Note that proteins carrying analogous mutations in NBD1, K218T, G217V, and a new double mutant K218T:A315T with severe defects in ATPase activity assembled in the presence of ATP even at low protein concentrations (Fig. 1*C* and data not shown).

The Relationship Between Hexamerization and ATP Hydrolysis in the NBD2 Mutants. Next, we asked if overcoming the assembly defect of NBD2 mutants would also overcome their ATP hydrolysis defects. At protein concentrations of 0.01 to 0.02 mg/ml and an ATP concentration of 5 mM, the K620T and G619V proteins hydrolyzed ATP at ≈1/20th the rate of WT Hsp104 (10). At

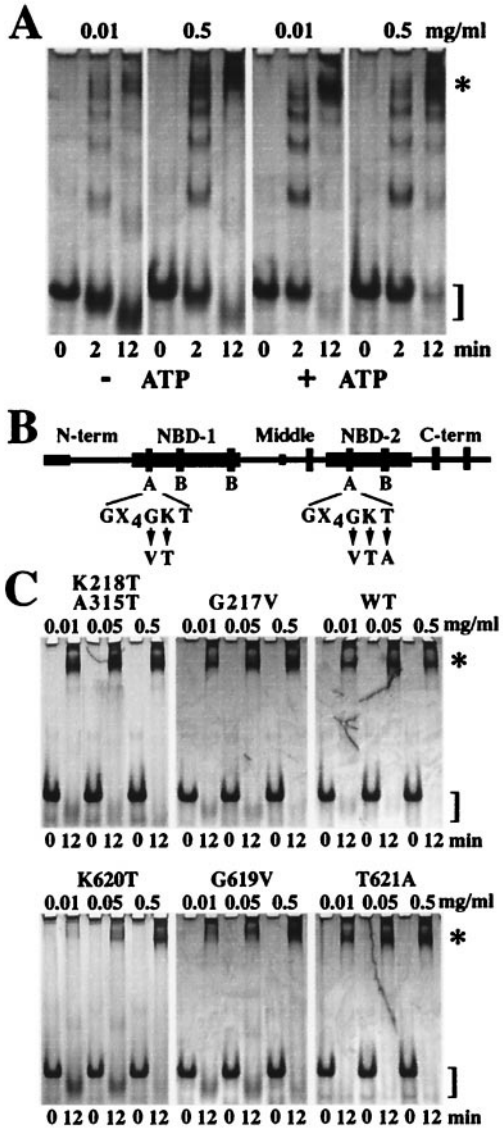

Fig. 1. Assembly of Hsp104 proteins. (*A*) Effect of high protein concentrations on the assembly of WT Hsp104 into hexamers. WT Hsp104 protein at different concentrations was incubated at 37°C with glutaraldehyde in the presence or absence of ATP for 2 or 12 min. Cross-linked proteins were separated on SDS/3.5% polyacrylamide gels and stained with silver. Intramolecular cross-links increased the mobility of monomers indicated by brackets. In the absence of ATP, most Hsp104 was unassembled when cross-linked at low protein concentrations but hexameric (indicated by asterisks) when cross-linked at high protein concentrations. In the presence of ATP, Hsp104 was hexameric at both protein concentrations. (*B*) General structural features of Hsp104 and the location of mutations used in this study. Sequence regions that are highly conserved in the HSP100 family, including the Walker A and B motifs, are indicated by thicker lines. The P-loop (Walker A) consensus sequences are given, and positions of mutations therein are indicated by arrows. (*C*) Effects of protein concentration on the assembly of mutant Hsp104 proteins. Mutant proteins (0.01, 0.05, or 0.5 mg/ml) were incubated with 5 mM ATP, cross-linked with glutaraldehyde for 0 or 12 min, and analyzed as above. The K218T:A315T and G217V mutants, like WT, hexamerize at all protein concentrations, but the K620T and G619V mutants hexamerize only at high protein concentrations. Unlike the other NBD2 mutants, the T621A mutant did not exhibit an assembly defect.

higher protein concentrations, the rate of ATP hydrolysis increased dramatically, reaching 40–80% of WT levels at 0.5 mg/ml (Fig. 2 and data not shown).

The two proteins that carried analogous P-loop substitutions

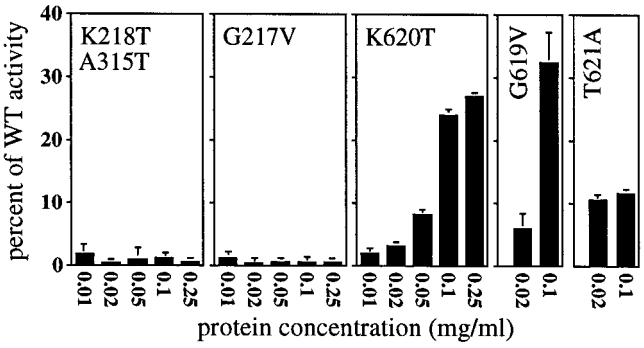

Fig. 2. Effects of protein concentration on the ATPase activity of mutant Hsp104 proteins. ATPase assays were performed with 5 mM ATP, using the Malachite Green colorimetric assay to measure released phosphate. The same buffer conditions were used as in cross-linking studies with different concentrations of Hsp104. The endpoints of reactions were varied to keep them in the linear range of the assay and subsequently adjusted to min^{-1}. Mutant activity is shown as the % of WT activity in the same experiment; mean and standard deviations are shown. WT Hsp104 released 0.67, 0.77, 1.10, 0.89, 1.02, and 0.80 nmol of P_i min^{-1} μg^{-1}, respectively, at 0.01, 0.02, 0.05, 0.1, 0.25, and 0.5 mg/ml. ATP hydrolysis by the K218T:A315T and G217V mutants was negligible at all protein concentrations, but the low hydrolysis by the K620T and G619V mutants increased more than 10-fold at high protein concentrations. No change occurred in the T621A mutant, which was oligomerized at all protein concentrations.

in NBD1, K218T and G217V (10) failed to hydrolyze detectable amounts of ATP at any protein concentration tested (Fig. 2 and data not shown). The same was true for the double mutant K218T:A315T. Another mutant, not previously described, carried a threonine to alanine substitution adjacent to the P-loop lysine in NBD2, T621A. In the presence of ATP, this mutant assembled into hexamers, even at low protein concentrations (Fig. 1*C*). Its ATPase activity was lower than WT protein but did not change at any of the concentrations tested (Fig. 2). These data suggest that the increase in ATP hydrolysis with the G619V and K620T proteins at high concentrations results from overcoming their assembly defects.

Kinetic Analysis of Mutant and WT Proteins. To determine what parameters of ATP hydrolysis are affected by assembly, the V_{max} and K_m values for ATP hydrolysis were determined at different protein concentrations. At the highest and lowest concentrations, linearity was lost over the broad range of ATP concentrations required for these assays, but at protein concentrations between 0.02 and 0.1 mg/ml, ATPase activity remained linear from 500 μM to 25 mM. As determined by cross-linking, most K620T or G619V protein was in the unassembled form at 0.02 mg/ml; most was in the hexameric form at 0.1 mg/ml (data not shown). For WT Hsp104 at 0.1 and 0.02 mg/ml, the K_m and V_{max} values were similar (Fig. 3; Table 1). For K620T, the V_{max} increased at the higher protein concentration. However, no further increase in V_{max} occurred when the protein concentration was further increased from 0.1 to 0.25 mg/ml (ATPase activity remained in the linear range for K620T at 0.25 mg/ml). That is, once this mutant protein assembled, V_{max} did not continue to increase as the protein concentration increased.

Because WT Hsp104 forms hexamers even at very low protein concentrations when ATP is present, we used a different experimental approach to examine the effects of assembly on ATP hydrolysis. As determined by glutaraldehyde cross-linking (Fig. 4*A*) and by sizing chromatography (J. R. Glover and S.L., unpublished observations), the assembly of WT Hsp104, in the presence of ATP, can be manipulated with the zwitterionic detergent CHAPS. WT Hsp104 assembles into hexamers in 2

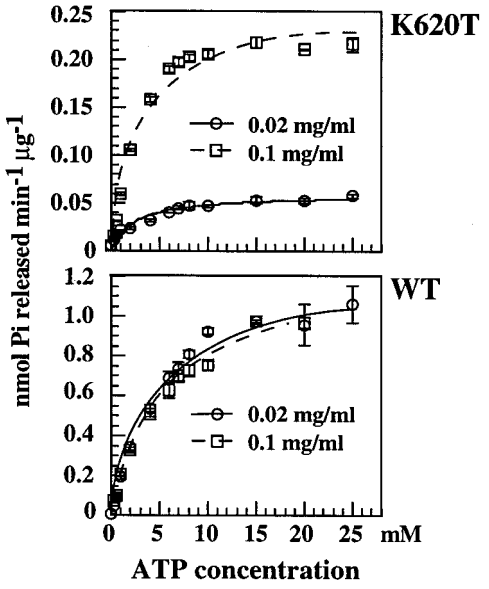

Fig. 3. Kinetic analysis of ATP hydrolysis. K620T and WT proteins at different protein concentrations. Phosphate released was determined after 4 min at the high protein concentration (0.1 mg/ml) and after 16 min at the low protein concentration (0.02 mg/ml) to ensure that reactions were within the linear range of the assay. Assays were performed in the same buffer as cross-linking studies (Fig. 1*A*) at 37°C. The rate of hydrolysis was calculated from the nanomoles of phosphate released by 0.5 μg of Hsp104 (0.02 mg/ml) during the first 5 min of incubation with ATP at various concentrations. K_m and V_{max} values were determined by least squares fitting to the Michaelis–Menten equation by using the KALEIDAGRAPH program (Synergy Software). Kinetic properties of the WT protein are similar at both protein concentrations, but for the K620T mutant V_{max} values increased 4-fold at the higher protein concentration.

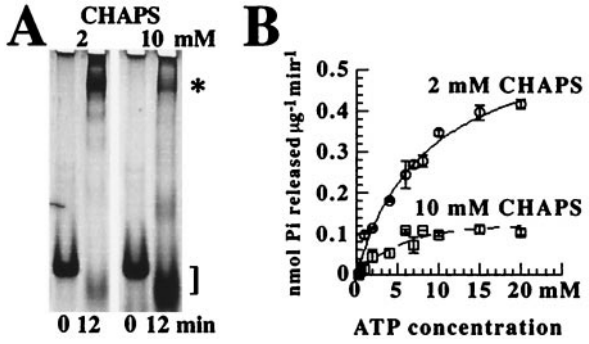

Fig. 4. Effects of the zwitterionic detergent CHAPS on the hexamerization and ATPase activities of WT Hsp104. (*A*) Cross-linking assays were performed as in Fig. 1*A*, in the presence of 2 mM or 10 mM CHAPS. Assembly was inhibited at the higher detergent concentration. (*B*) Kinetic analysis of WT Hsp104 in the presence of CHAPS. WT Hsp104 (0.02 mg/ml) was incubated for 16 min and K_m and V_{max} values were calculated as in Fig. 3. In different experiments, the V_{max} for ATP hydrolysis was 2- to 4-fold less in 10 mM CHAPS (hexameric, dashed line) than in 2 mM CHAPS (unassembled), whereas K_m was changed less than 2-fold.

mM CHAPS (below the critical micelle concentration) but not in 10 mM CHAPS (just above the critical micelle concentration). When assembly of WT Hsp104 was inhibited with CHAPS, ATP hydrolysis was reduced, albeit not as strongly as in the unassembled K620T (Fig. 4*B*). Notably, however, reducing the CHAPS concentration to 2 mM to promote assembly had the same effect as increasing the protein concentration of the K620T protein; it increased the rate of hydrolysis by increasing V_{max} severalfold. Together, these data indicate it is the rate of turnover that is most affected when Hsp104 assumes a hexameric form rather than the affinity for ATP (at least at the site, or sites, responsible for the majority of ATP hydrolysis under these conditions).

ATP Hydrolysis Is Cooperative in Hexameric Hsp104. One mechanism by which the interaction between Hsp104 subunits might increase the rate of ATP hydrolysis is through cooperativity, with ATP hydrolysis at one site increasing the likelihood of hydrolysis at another. Positive cooperativity should produce a Hill coefficient greater than 1. A series of kinetic assays were performed over a broad range of ATP concentrations (10 μM to 40 mM) at a concentration where both WT Hsp104 and K620T were hexameric. For each protein, the Hill coefficient was repeatedly between 1.5 and 2 (Fig. 5). Apparently, subunit interactions in Hsp104 produce cooperativity in ATP hydrolysis, identifying at least one factor that influences the rate of hydrolysis upon assembly. They further indicate that oligomerization restores some of the basic parameters of cooperative hydrolysis in the K620T mutant.

The Role of Cooperativity in Hsp104 Function. To investigate the importance of cooperative subunit interactions *in vivo*, we sought conditions by which it could be blocked in a biological context. The mutant K218T:A315T was the logical choice to pursue this aim. It virtually eliminated ATP hydrolysis, yet it efficiently assembled into hexamers, both on its own and in the presence of WT Hsp104 protein (by glutaraldehyde cross-linking, data not

Table 1. K_m and V_{max} values calculated for the K620T and WT proteins at two different protein concentrations

Protein	Conc., mg/ml	K_m, mM	V_{max}, nmol $min^{-1}\mu g^{-1}$
K620T	0.02	2.3	0.06
	0.10	2.5	0.25
WT	0.02	4.8	1.25
	0.10	5.9	1.28

V_{max} is in nmol of P_i released per min per μg of protein.

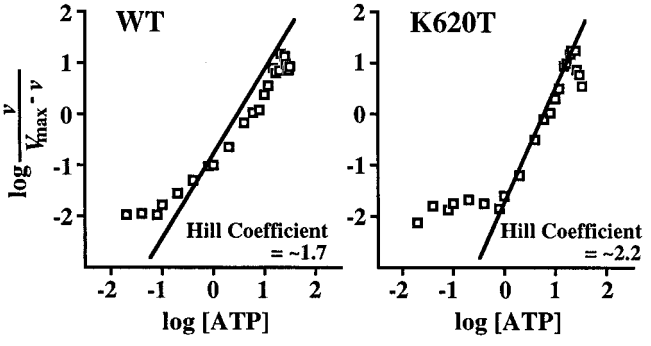

Fig. 5. Cooperativity of ATP hydrolysis. Kinetic analysis of K620T mutant and WT Hsp104 (0.25 mg/ml) ATP hydrolysis at ATP concentrations ranging from 20 μM to 32 mM. ATP hydrolysis was cooperative, yielding a Hill coefficient of ≈2.2 (standard error 0.2) for K620T and ≈1.7 (standard error 0.2) for WT. The rate of hydrolysis was calculated during the first 4 min of incubation with ATP for WT and 7 min for K620T to ensure that reaction products were measurable within the linear range of the assay. The Hill coefficients were calculated by fitting the nonlinear curves from plotting millimolar ATP vs. nmol of P_i released min^{-1} μg^{-1} to the Michaelis–Menten equation by using the allosteric kinetic equation of the GRAFIT 4 graphics program (Erithacus Software) and were similar to those calculated from the slope of the linear Hill plots shown.

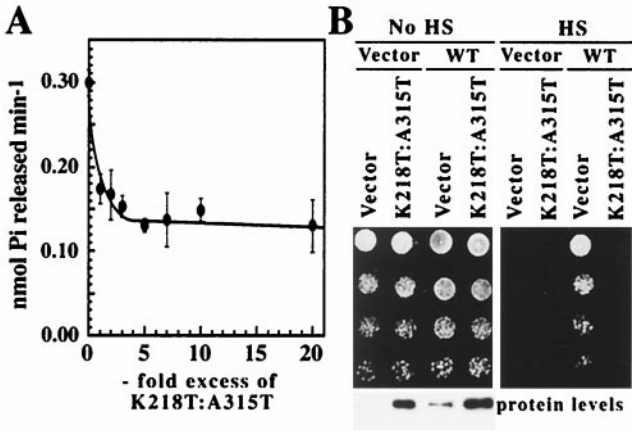

Fig. 6. Effect of the K218T:A315T mutant protein on WT Hsp104. (*A*) Different concentrations of K218T:A315T were added to a fixed quantity of WT protein (0.5 μg per reaction, 0.02 mg/ml), and phosphate release was determined as in Fig. 2. K218T:A315T inhibited WT ATPase activity, reaching maximum inhibition at ≈5-fold excess. (*B*) Effect of the K218T:A315T protein on the biological activity of WT Hsp104. Cells had an integrated, constitutive K218T:A315T expression plasmid or the vector control and an estradiol-inducible WT *HSP104* expression plasmid or the vector control. WT Hsp104 was induced to <20% the level of K218T:A315T for 2 h at 25°C. Levels of Hsp104 protein before heat shock are shown for equal numbers of cells beneath the NO HS panel. Cells were serially diluted (5-fold each step) and plated to determine survival at 25°C before (No HS) or after (HS) a 4-min heat shock at 50°C. Constitutive expression of K218T:A315T had no adverse affect on growth in the absence of heat shock but blocked the increase in survival produced by WT Hsp104 after heat shock.

shown). Coassembly with K218T:A315T should block cooperative ATP hydrolysis by WT Hsp104. Thus, the rate of ATP hydrolysis in mixed oligomers of K218T:A315T and WT Hsp104 should decrease as the concentration of K218T:A315T is increased. Indeed, addition of increasing amounts of K218T:A315T to a fixed quantity of WT protein (0.5 μg) decreased, but did not eliminate, ATP hydrolysis (Fig. 6*A*). Inhibition plateaued at a mutant to WT ratio of 5:1 (for which the rate of hydrolysis is similar to that obtained by including CHAPS in the reactions to prevent oligomerization). This was not due to competition for substrate, as ATP was in excess, nor was it due to the change in protein density because (*i*) the addition of BSA in place of K218T:A315T did not inhibit hydrolysis by Hsp104, (*ii*) the addition of WT Hsp104 in place of K218T:A315T increased hydrolysis in a linear manner, and (*iii*) the addition of K620T to WT Hsp104 increased the total ATPase activity in a nearly additive manner (data not shown). Thus, we conclude that excess K218T:A315T blocks cooperative ATP hydrolysis by WT Hsp104. At its maximum effect, this block in cooperativity is not so strong as to eliminate hydrolysis; it simply reduces it approximately 2-fold.

Next, we examined the effects of excess K218T:K315T on WT Hsp104 *in vivo*. We have previously shown that K218T is unable to provide thermotolerance in the absence of WT Hsp104 but has no deleterious effects on growth (20). Cells coexpressing K218T and WT Hsp104 at similar levels had normal levels of thermotolerance (D. A. Parsell and S.L., unpublished observations). However, very little functional Hsp104 is required for thermotolerance (28, 29). Thus, those Hsp104 hexamers that do not contain sufficient K218T:A315T to block cooperative ATP hydrolysis by WT Hsp104 could well be sufficient for thermotolerance. In light of our observation that a 5-fold excess of K218T:A315T is required to maximize the effects of this mutant on the ATPase activity of WT Hsp104 *in vitro* (Fig. 6*A*), we engineered the expression of K218T:A315T such that the K218T:A315T/WT ratio would be ≈5 *in vivo*. In this case, thermotolerance was reduced dramatically. In the absence of heat shock, high levels of the K218T:A315T protein had no adverse effect on growth in either cells expressing WT Hsp104 or cells carrying an *HSP104* deletion (Fig. 6*B*, NO HS; data not shown). Moreover, the K218T:A315T mutant did not reduce the low level of residual thermotolerance that is present in *hsp104* deletion cells after a conditioning preheat treatment (data not shown). Because the K218T:A315T mutant had no deleterious effects on its own, we suggest that the decrease in thermotolerance it produces in the presence of WT Hsp104 is due to the ability of this mutant to alter the activity of the WT protein in mixed oligomers.

The K620T mutant was also assayed *in vivo*. In the absence of WT Hsp104, overexpressing K620T had no deleterious effects on growth. However, overexpression of K620T in the presence of WT Hsp104 caused some growth inhibition at normal temperatures (data not shown), preventing testing of the effect of K620T on the thermotolerance function of WT Hsp104. Nonetheless, it provides independent evidence of the importance of subunit interactions in regulating Hsp104 function *in vivo*.

Discussion

We have shown that the activities of Hsp104 are strongly influenced by subunit interactions, both *in vitro* and *in vivo*. In experiments where the hexameric form of WT Hsp104 was controlled by the detergent CHAPS or the hexameric form of NBD2 mutants was controlled by protein concentration, ATP hydrolysis was higher in the assembled state than in the nonassembled state by about 4-fold. In both cases, this was primarily due to an increase in V_{max}. We have also determined that hydrolysis is cooperative in Hsp104, thereby identifying a contributing factor for increased hydrolysis in the assembled state. Our data further suggest that cooperativity and subunit interactions are vital to the thermotolerance function of Hsp104 *in vivo*.

Earlier work had indicated functional distinctions between the two NBDs of Hsp104 (10, 11); NBD1 mutations virtually eliminate hydrolysis without affecting assembly, whereas analogous mutations in NBD2 severely affect assembly while reducing hydrolysis ≈10-fold. We now find that high protein concentrations overcome the assembly defects of NBD2 mutants and at the same time increase the rate of ATP hydrolysis to 40–80% that of the WT. Together with earlier data, these observations argue strongly that NBD1 is responsible for most of the ATPase activity of the protein, whereas nucleotide binding or hydrolysis at NBD2 regulates structural parameters that affect NBD1 activity (for example, in response to substrate and cofactor interactions). It remains possible that both sites contribute more equally to hydrolysis. If so, the ATPase activities of the two domains must be coupled in such a way that the basal activity of NBD2 strongly depends on the activity of NBD1, but the basal activity of NBD1 is relatively independent of NBD2 activity when the protein is assembled.

If most ATP hydrolysis occurs at NBD1, then the Hill coefficient of ≈2 likely reflects cooperative hydrolysis between NBD1 subunits in the hexamer rather than between NBD1 and NBD2. This idea is further supported by the allosteric interactions we observed by inhibiting WT Hsp104 ATPase activity in mixed oligomers, an effect that has also been observed for the related ClpA protein (22). Furthermore, a residue in the C terminus that is highly conserved among all HSP100 proteins was shown to interact with a conserved P-loop residue of an adjacent subunit in oligomers of HslU, another member of the HSP100 protein family (30). And in separate work, we have found that the affinity of NBD2 for ATP is 100-fold greater than that of NBD1 (D. Hattendorf and S.L., unpublished results). Most of the kinetic parameters

of ATP hydrolysis examined here reflect the effects of ATP binding to the lower affinity site, NBD1.

Unlike HslU, which has only one NBD and a C-terminal region, Hsp104 has two NBDs flanked by N-terminal, middle, and C-terminal regions. Thus, a large number of interactions between domains and subunits is possible. Indeed, we have found that the rate of ATP hydrolysis increases substantially when polypeptides are bound to the C terminus of Hsp104 and that the middle region of Hsp104 appears to play an important role in mediating changes in NBD1 activity in response to C-terminal interactions (A. Cashikar, E.C.S., D.M.W., and S.L., unpublished data). This middle region of Hsp104 and other HSP100 proteins that function in thermotolerance is long, whereas that of the HSP100/Clp proteins that function in proteolysis is much shorter or absent; thus, the middle region's effects on the ATPase reaction cycle may distinguish these functions. In separate work, we have found that the rate of ATP hydrolysis in assembled Hsp104 is increased in low salt buffers (10). In that case, the increase in hydrolysis specifically reflected a change in the K_m measured by ATP hydrolysis. In contrast, the effects of oligomerization reported here specifically affect V_{max}. Taken together, this work suggests that the two NBDs of Hsp104 work together in a complex cycle that involves multiple ATP binding and hydrolysis states, which may be influenced by many interactions within the hexamer and with substrates.

In addition to demonstrating that cooperativity and oligomeric contacts influence the parameters of ATP hydrolysis by Hsp104, we have established that these contacts profoundly influence the function of the protein in living cells. Unlike many other chaperones, WT Hsp104 is not toxic to growth even when it is overexpressed at very high concentrations (28, 29). The fact that K620T slightly inhibits growth at normal temperatures, but only in the presence of WT protein, suggests correct subunit interactions are normally required to control the biological activities of Hsp104. Further, when the K218T:A315T mutant was expressed in the presence of WT Hsp104 at a ratio equivalent to that which maximally inhibits WT Hsp104 ATP hydrolysis *in vitro*, it strongly inhibited the thermotolerance function of WT Hsp104 *in vivo*. Yet, the K218T:A315T mutant had no toxic effects on its own. The observations reported here provide a biological confirmation of earlier biochemical suggestions that the two NBDs of Hsp104 have different functional roles. They also indicate that correct subunit interactions in the hexameric form are vital for thermotolerance function. This was by no means a forgone conclusion. K218T:A315T reduced the free-running ATPase activity of WT Hsp104 in mixed oligomers by only ≈2-fold, and we have previously shown that a very small quantity of Hsp104 is sufficient for thermotolerance (28). Yet, even larger quantities of WT Hsp104 are insufficient for thermotolerance when cooperative subunit interactions are perturbed by the induction of K218T:A315T. The conditional expression of this protein will provide an important tool for the analysis of Hsp104 function in the maintenance of yeast prions and the aggregation-prone human disease proteins such as huntingtin.

We thank E. Taylor, J. Westley, J. Glover, and A. Cashikar for helpful discussions. We thank J. Clos for the pJC45 vector and O. Fayet for strain BL21[DE3](pAPlacIQ). We thank D. Parsell for construction of some mutants and L. Rosman and L. Loftus for technical assistance. We also thank A. Cashikar, D. Hattendorf, M. Ramakrishnan, and T. Serio for critical reading of the manuscript. This work was supported by Department of Energy Grant FG02-95ER20207. E.C.S. was supported by Public Health Service Grant 6 T32 GM07183-19; C.Q. is supported by the Boehringer Ingelheim Fonds, and the work presented is part of her doctoral thesis.

1. Schirmer, E. C., Glover, J. R., Singer, M. A. & Lindquist, S. (1996) *Trends Biochem. Sci.* **21,** 289–296.
2. Wickner, S., Maurizi, M. R. & Gottesman, S. (1999) *Science* **286,** 1888–1893.
3. Hwang, B. J., Woo, K. M., Goldberg, A. L. & Chung, C. H. (1988) *J. Biol. Chem.* **263,** 8727–8734.
4. Katayama, Y., Gottesman, S., Pumphery, J., Rudikoff, S., Clark, W. P. & Maurizi, M. R. (1988) *J. Biol. Chem.* **263,** 15226–15236.
5. Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B. & Chung, C. H. (1992) *J. Biol. Chem.* **267,** 20429–20434.
6. Wilson, S. A., Williams, R. J., Pearl, L. H. & Drew, R. E. (1995) *J. Biol. Chem.* **270,** 18818–18824.
7. Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C. & Zylicz, M. (1995) *EMBO J.* **14,** 1867–1877.
8. Shin, D. H., Yoo, S. J., Shim, Y. K., Seol, J. H., Kang, M.-S. & Chung, C. H. (1996) *FEBS Lett.* **398,** 151–154.
9. Turgay, K., Hamoen, L. W., Venema, G. & Dubnau, D. (1997) *Genes Dev.* **11,** 119–128.
10. Schirmer, E. C., Queitsch, C., Kowal, A. S., Parsell, D. A. & Lindquist, S. (1998) *J. Biol. Chem.* **273,** 15546–15552.
11. Parsell, D. A., Kowal, A. S. & Lindquist, S. (1994) *J. Biol. Chem.* **269,** 4480–4487.
12. Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K. & Steven, A. C. (1995) *J. Mol. Biol.* **250,** 587–594.
13. Kessel, M., Wu, W.-f., Gottesman, S., Kocsis, E., Steven, A. C. & Maurizi, M. R. (1996) *FEBS Lett.* **398,** 274–278.
14. Sanchez, Y. & Lindquist, S. L. (1990) *Science* **248,** 1112–1115.
15. Sanchez, Y., Taulien, J., Borkovich, K. A. & Lindquist, S. (1992) *EMBO J.* **11,** 2357–2364.
16. Serio, T. R. & Lindquist, S. L. (2000) *Trends Cell Biol.* **10,** 98–105.
17. Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J. S., Dalrymple, B., Kuramitsu, H., Shiroza, T. & Foster, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3513–3517.
18. Lindquist, S. & Schirmer, E. C. (1999) in *Molecular Chaperones and Folding Catalysts: Regulation, Cellular Function and Mechanisms*, ed. Bukau, B. (Harwood, Amsterdam), pp. 347–380.
19. Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982) *EMBO J.* **1,** 945–951.
20. Parsell, D. A., Sanchez, Y., Stitzel, J. D. & Lindquist, S. (1991) *Nature (London)* **353,** 270–273.
21. Saraste, M., Sibbald, P. R. & Wittinghofer, A. (1990) *Trends Biochem. Sci.* **15,** 430–434.
22. Singh, S. K. & Maurizi, M. R. (1994) *J. Biol. Chem.* **269,** 29537–29545.
23. Seol, J. H., Baek, S. H., Kang, M. S., Ha, D. B. & Chung, C. H. (1995) *J. Biol. Chem.* **270,** 8087–8092.
24. Louvion, J.-F., Havaux-Copf, B. & Picard, D. (1993) *Gene* **131,** 129–134.
25. Schirmer, E. C. & Lindquist, S. (1998) *Methods Enzymol.* **290,** 430–444.
26. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97.
27. Schirmer, E. C., Lindquist, S. & Vierling, E. (1994) *Plant Cell* **6,** 1899–1909.
28. Lindquist, S. & Kim, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5301–5306.
29. Sanchez, Y., Parsell, D. A., Taulien, J., Vogel, J. L., Craig, E. A. & Lindquist, S. (1993) *J. Bacteriol.* **175,** 6484–6491.
30. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D. & Huber, R. (2000) *Nature (London)* **403,** 800–805.